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Decreased wound neutrophils and indiscrete margination in the pathogenesis of wound infection

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To assess the pathogenesis of increased susceptibility to infection and septic death in a rat model, neutrophils (PMNs) in the wound, circulating PMNs, and their *in vivo* activity were evaluated after 30% and 60% burns. Eight hours after injury there were twice as many PMNs in the wounds of rats that sustained 30% compared with 60% burns. There was no difference between these two groups in the number of circulating PMNs at 2, 4, 6, and 8 hours after injury. *In vivo* evaluation of PMN response to infusion of F-Met-Leu-Phe revealed that circulating PMNs were more sensitive 4 hours after 60% burns compared with sham burns. At this time PMNs were found to be less sensitive to zymosan-activated serum infusion after 30% burns compared with sham burns. However, the PMNs in rats with 30% burns were more sensitive to this stimulus than were PMNs in rats with 60% injuries. Thus rats with greater injury, known to be more susceptible to wound infection, have fewer PMNs in their wounds 8 hours after injury. This is preceded by an increased sensitivity of PMNs *in vivo* to bacterial chemotactic factor and a relative increase in sensitivity to wound factors. This unusual finding implicates indiscrete margination as a factor in the pathogenesis of infection.

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THE CLINICAL OBSERVATION that susceptibility to infection is directly related to extent of injury is supported by laboratory studies that show a graded depression of cellular,^{1,2} humoral,^{3,4} and nonspecific^{5,6} immune response after injury. Nevertheless, variation in the extent of injury, the lack of availability of the patient for study immediately after injury, and the invasive nature of some testing have impeded strictly controlled study of the acute response to injury. Therefore a rat model of burn injury⁷ that parallels the clinical situa-

tion in human patients⁸ in which a predictable, although unpredictable for a given patient, increase in mortality rates from sepsis occurs with increasing extent of injury has been developed. Rats that sustain a 60% (30% partial plus 30% full thickness) total body surface area (TBSA) burn develop bacterial invasion of the partial-thickness burn after surface inoculation with *Pseudomonas aeruginosa* strain 59-1244. However, rats that are treated in the same manner except that they sustain only 30% TBSA partial-thickness burns do not develop bacterial invasion of the wounds. Mortality rates for sepsis in rats that sustain 60% TBSA burns are significantly higher than those in rats that sustain 30% TBSA burns.

The current study was designed to evaluate and compare the early postinjury neutrophil response after these injuries. Although no difference was found in the number of circulating neutrophils at various times after 30% or 60% TBSA burns, the wounds in rats that sustained the smaller burn contained twice as many neutrophils 8 hours after injury. In contrast, *in vivo* response of neutrophils to chemotactic factor infusion was found to be greater in rats with larger burns 4 hours after injury. These findings suggest that

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In concluding this research, we adhered to the *Guide for the Care and Use of Laboratory Animals*, as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

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increased susceptibility to wound infection may be due to a decreased number of neutrophils in the wound that is a consequence of indiscrete margination of sensitized neutrophils in distant capillary beds.

MATERIAL AND METHODS

Sprague-Dawley rats, weighing 350 gm, were used in all experiments. Each rat received 0.5 ml of pentobarbital sodium (25 mg/kg) intraperitoneally before burn injury. Rats were placed in molds to expose 30% of the TBSA to water at a temperature of 95° C.⁹ Contact of the rat's dorsal surface with the water for 2 or 10 seconds led to partial- or full-thickness injury, respectively. Two-second exposure of the ventral surface produced full-thickness injury. In each case after dorsal injury the rat received 15 or 30 ml of saline solution when the total extent of injury was 30% or 60%, respectively. The rat was repositioned to allow ventral exposure to water at 95° C or nothing (sham). The desired surface area and depth of injury were obtained consistently as described previously.⁷ Depth of injury in surviving rats was confirmed by clinical evaluation of wounds 2 to 4 weeks after injury. Skin biopsy specimens were taken after pentobarbital anesthesia by sharp dissection and fixed in 10% buffered formalin. Depth of injury was confirmed by evaluation of sections stained with hematoxylin-eosin. All neutrophils and vessels immediately adjacent to the dermal surface of the panniculus carnosus were enumerated in 30 fields at 450-fold magnification in Giemsa-stained tissue sections mounted on label-blinded slides. To eliminate differences in skin thickness caused by edema as a variable, neutrophil counts were expressed as number of neutrophils per vessel.

In experiments requiring sequential blood sampling or systemic infusions, the rats were cannulated according to the method of Harms and Ojeda¹⁰ on the day before study. Each rat was anesthetized with 0.05 ml fentanyl citrate (Innovar) by intramuscular injection, and silicone rubber medical-grade tubing was passed from the external jugular vein into the superior vena cava. The proximal portion of the catheter was tunneled through the subcutaneous tissue and brought out through a stab incision in the posterior aspect of the neck. The catheter was irrigated with saline solution containing 1 U heparin/ml and sealed between uses. One hundred to 500 μ l blood samples were drawn into 3.8% sodium citrate in a ratio of 10 to 1, respectively. No more than five blood samples were drawn from any one rat.

In vivo response of neutrophils to infusion of saline solution, N-formyl-L-methionyl-leucyl-L-phenylala-

nine (FMLP), or zymosan-activated rat serum (ZAS) was determined by enumeration of circulating neutrophils before and after the infusion of 0.5 ml of the desired solution. Catheters were flushed with 100 μ l of saline solution after all infusions, and 50 μ l of whole blood was withdrawn and discarded immediately before blood sampling. Preliminary experiments indicated that response to FMLP infusion could be reproducibly measured 3 minutes after infusion. As described previously by Gilbertsen et al.,¹¹ a secondary neutrophilia was often seen after ZAS infusion and therefore the neutropenia response to ZAS was measured at the earlier time of 1 minute after infusion. Response to infusion was expressed as the percent of preinfusion neutrophils in central venous blood at the indicated time after infusion. Cell counts and differentials were performed by standard methods on a ZBI-Coulter Counter (Coulter Electronics Inc., Hialeah, Fla.) and whole blood smears, respectively.

ZAS was prepared by a previously described method.¹¹ Thirty milliliters of whole blood was obtained by aortic puncture from rats after methoxyflurane (Penthrane) anesthesia. Serum was incubated with boiled zymosan (10 mg/ml serum) for 60 minutes at 37° C and centrifuged at 1500 \times g for 10 minutes. The supernatant was heat inactivated for 30 minutes at 56° C, centrifuged at 1500 \times g for 10 minutes, and stored in 1 ml aliquots in polypropylene tubes at -70° C. FMLP stock solutions were prepared immediately before use by solubilization of FMLP in 50 μ l of dimethyl sulfoxide (DMSO) followed by the addition of sufficient 0.9% NaCl to yield a solution of 1 mg/ml. The minimum dilution of DMSO was 1:80 in all experiments and control infusion of 0.9% NaCl containing this amount of DMSO had no effect on neutrophil counts.

Data are expressed as means \pm SEM. The unpaired Student *t* test was used to compare wounds. Multiple comparisons were performed after analysis of variance with the Student-Newman-Keuls test.¹² Differences were considered to be significant when *p* < 0.05.

RESULTS

In preliminary experiments the time-related accumulation of neutrophils in wounds was determined in skin biopsy specimens from five rats each at 1, 2, 3, 4, 6, or 8 hours after 30% partial-thickness burn injury. The results from counting 10 high-power fields (HPF) in each biopsy specimen indicated that there was a progressive increase in marginating neutrophils during the first 3 hours after injury. By 4 hours, infiltration of neutrophils into the wound became prominent and 8

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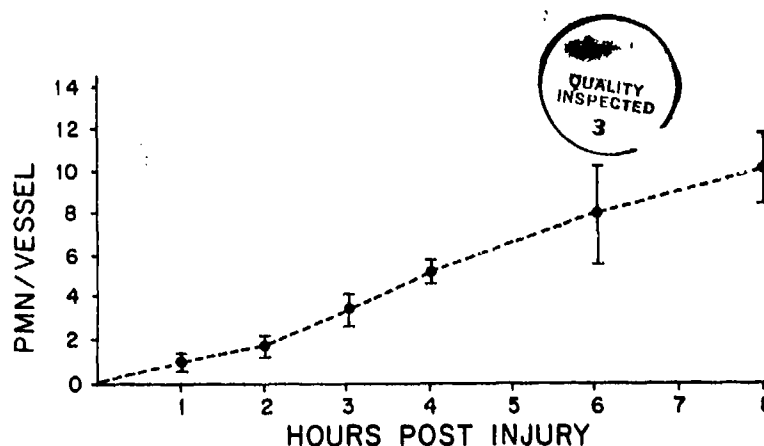


Fig. 1. Time-related accumulation of neutrophils in wounds after 30% TBSA partial-thickness burn.

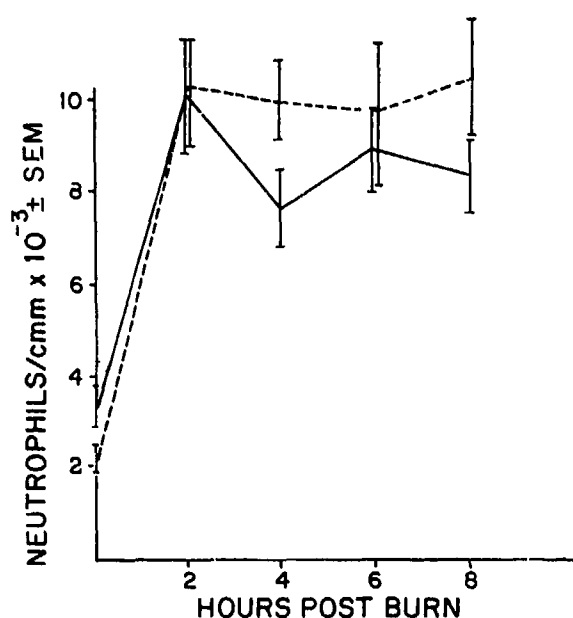


Fig. 2. Time-related change in number of neutrophils in the central venous blood of rats after 30% partial-thickness (---) and 30% full-thickness (—) burn. There was no significant difference between groups at any time.

hours after injury the majority of the neutrophils were found in the vessels (Fig. 1). In some cases the inflammatory response varied with regard to intensity within individual biopsy specimens, and therefore all later biopsy specimens were evaluated by counting 30 HPF.

Since neutrophil infiltration into the wounds became prominent 4 hours after injury, this time and 8 hours after injury were selected to compare the inflammatory responses in the partial-thickness wounds of rats with either 30% partial-thickness injury or that injury plus an additional 30% full-thickness burn. Four hours after injury there were no significant differences

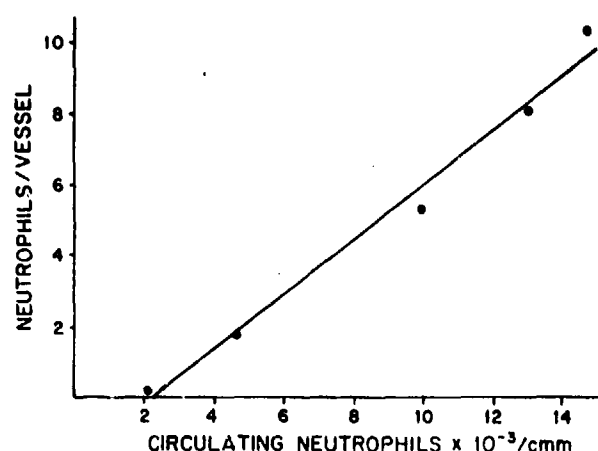


Fig. 3. Comparison of the number of neutrophils in venous blood and in wounds at various times after 30% partial-thickness burn.

between the 30% (n = 5) and 60% (n = 5) burned rat's partial-thickness wounds with regard to number of neutrophils per vessel (Table I). However, by 8 hours after injury there were significantly fewer neutrophils in the wounds of the rats that sustained 60% burns compared with those that sustained 30% burns. That there were fewer neutrophils 8 hours after burn in the wounds of rats that sustained 60% injury compared with those with 30% burns was confirmed in a repeat experiment. The combined data from all 8-hour experiments showed that there were more than two times as many neutrophils in the wounds of the 30% burns (n = 10) compared with the 60% (n = 10) burns (Table I).

To determine if the difference in the number of neutrophils in the wounds was due to a difference in the number of neutrophils in the circulation, total neutrophil counts were determined before and 2, 4, 6, and 8 hours after these injuries. Although total neutro-

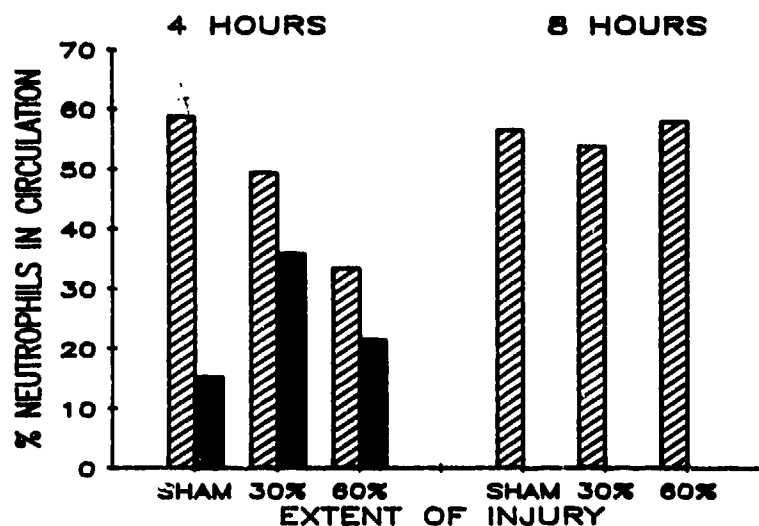


Fig. 4. The in vivo response of neutrophils to the intravenous infusion of 5.0 nmol of FMLP (crosshatch bar) or 200 μ l of ZAS (solid bar) after sham, 30% partial-thickness, and 30% full-thickness burns. The results are expressed as the percent of the preinfusion neutrophil count 3 minutes (FMLP) or 1 minute (ZAS) after infusion. The response was determined 4 (FMLP and ZAS) and 8 (FMLP) hours after injury.

Table I. Time-related accumulation of neutrophils in wounds after 30% and 60% burns in rats

Injury (%)	Time of biopsy (hr)	Neutrophils/vessel (\pm SEM)
30*	4	1.36 \pm 0.32
60	4	1.78 \pm 0.36
30	8	3.54 \pm 0.59
60	8	1.61 \pm 0.42 [†]

*Thirty percent partial-thickness, 60% = 30% partial thickness plus 30% full thickness injury.

[†] $p < 0.02$.

phil count appeared to be slightly lower in rats with 60% TBSA burns 4 hours after injury, there was no significant difference between rats that sustained 60% ($n = 5$) or 30% ($n = 5$) burns at any time (Fig. 2). In an additional experiment to ascertain the relationship between the accumulation of neutrophils and the number of circulating neutrophils, neutrophil counts were determined on blood drawn from each of five rats 2, 3, 4, 6, and 8 hours after 30% partial-thickness burn. The number of circulating neutrophils was found to increase in parallel ($r^2 = 0.984$; $p < 0.001$) with the number of neutrophils in 30% partial-thickness burn wounds (Fig. 3).

Since the number of circulating neutrophils was the same after 30% and 60% burns, the in vivo responsiveness of circulating neutrophils to infusion of chemotactic factors was evaluated. In a preliminary experiment,

normal rats were cannulated and 24 hours later infused with FMLP. There was a dose-related response with 3-minute neutrophil counts of $132.5\% \pm 17.6\%$ ($n = 3$), $46.2\% \pm 7.1\%$ ($n = 3$), and $9.9\% \pm 4.43\%$ ($n = 3$) of normal after infusion of 0.5, 5.0, and 50 nmol of FMLP, respectively. Studies of rats that sustained injury were performed with the dose (5.0 nmol) that gave the intermediate response. The results from the study of nine rats each at 4 hours after sham, 30% partial, or 30% partial plus 30% full-thickness burns indicated that neutrophils in rats with 60% burns ($33.6\% \pm 6.7\%$ in the circulation at 3 minutes) were significantly ($p < 0.05$) more sensitive than were sham-injured rats ($59\% \pm 4.9\%$). The percent of neutrophils in the circulation in rats that sustained 30% burns ($49.6\% \pm 10.1\%$) was not different from sham or 60% burn groups. Six additional rats in each of these groups were evaluated 8 hours after injury. No difference was found among the sham ($56.7\% \pm 3.6\%$), 30% ($54.0\% \pm 8.2\%$), and 60% ($58.1\% \pm 3.2\%$) injury groups (Fig. 4).

Based on the finding of a significant increase in in vivo sensitivity of neutrophils to FMLP infusion 4 hours after 60% injury, further studies with ZAS infusion were performed at this time. The dose of ZAS and timing of sampling were based on a dose-response study in normal rats that showed that 2.1%, 63.2%, and 98.7% of the preinfusion neutrophils were in the peripheral blood 1 minute after infusion of 300, 100, or 50 μ l of ZAS, respectively. After infusion of 200 μ l of

ZAS into each of eight rats that sustained sham, 30% partial, or that injury plus a 30% full-thickness burn, $15.5\% \pm 3.5\%$, $36.1\% \pm 2.8\%$, and $21.7\% \pm 3.9\%$ of the neutrophils were in the circulation, respectively (Fig. 4). The differences between sham and 30% and 30% and 60% groups were significant ($p < 0.05$).

Urine output and weight changes in the preburn and postburn period were evaluated in 30% partial-thickness ($n = 5$) and 30% partial plus 30% full-thickness ($n = 5$) injured rats. There was no difference in urine output or weight between the groups at 4, 8, and 24 hours and the subsequent 3 days after injury. Although the urine output dropped during the 4 hours immediately after injury in both groups, no rat had a urine output of less than 0.35 ml/kg/hr at any time. In an additional study, erythrocyte counts increased to $135\% \pm 4.9\%$ and $124\% \pm 4.3\%$ of normal values 2 hours after 30% and 60% burns, respectively. This difference was not significant and there were no significant differences between the groups as counts decreased at 4, 6, and 8 hours after injury.

DISCUSSION

The time-dependent development of the acute inflammatory response in the wounds of rats with 30% TBSA partial-thickness burns was defined in terms of neutrophil infiltration and margination (Fig. 1). After an early phase of neutrophil margination (1 to 4 hours), neutrophil infiltration into the tissue became prominent and increased during the subsequent 4 hours of study. When the circulating neutrophils were enumerated during this time period, a linear correlation between the number of circulating and wound neutrophils was observed (Fig. 3). Although such findings suggest that neutrophils accumulate in wounds in a passive and time-dependent manner, the additional studies indicating that extent of injury modulates neutrophil infiltration support the process as being active. In those studies the number of neutrophils in the wounds of rats that sustained 30% burns was twice as high 8 hours after injury as the number found in rats with 60% burns (Table I). However, the circulating neutrophil counts were the same during the first 8 hours after 30% and 60% injuries. These studies do not eliminate the possibility that neutrophil accumulation in the wound may be modulated by an active process of the vascular endothelium rather than on the neutrophil.

The implication that neutrophil response is modulated by injury is well documented in *in vitro* studies showing decreased chemotaxis of neutrophils^{5,13} caused a variety of mechanisms such as the presence of

inhibitors,¹⁴ degranulation,⁵ and deactivation.¹⁵ Furthermore, *in vivo* studies done in patients who sustained burns have suggested that neutrophil response to an iatrogenic wound is inversely proportional to the extent of injury.¹⁶ To assess the response of neutrophils as it relates to the extent of injury in the present study, a new method was applied. This *in vivo* evaluation allowed mediators of inflammation such as corticosterone,¹⁷ epinephrine,^{18,19} products of arachidonic acid metabolism,²⁰ and histamine²¹ to continue to impinge on neutrophils. The preliminary experiments in normal rats confirmed a previous report¹¹ that margination of rat neutrophils occurs in a dose- and time-related manner after intravenous injection of FMLP or ZAS. In contrast to previous reports of depressed neutrophil chemotaxis after injury, the present study showed that neutrophils were more sensitive to the bacterial-derived chemotactic factor, FMLP, 4 hours after 60% injury as compared with sham injury. At this time, however, circulating neutrophils in rats with 60% burns were not more sensitive (if anything, less sensitive) to ZAS infusion than were those in sham-injured rats (Fig. 4). This inconsistent response to the two chemotactic factors is likely based on a differential availability of receptors on the neutrophil surface. Complement activation with generation of fragments of C5, C5a, and C5a des arg has been documented after burns in patients²² and in rat²³ and mouse²⁴ models. Furthermore, *in vitro* studies document that exposure of neutrophils to C5a/C5a des arg-containing solutions²⁵⁻²⁷ or ZAS¹⁵ results in an increased number of receptors for FMLP. Indeed, others¹⁵ have reported an increased number of FMLP receptors on but a depressed response to ZAS *in vitro* in neutrophils obtained from injured patients who had products of complement activation in their serum. Therefore the data in the present *in vivo* study provide support on a functional basis for previous *in vitro* studies that suggest that neutrophils may be deactivated for C5a/C5a des arg after injury but continue to maintain²⁸ or increase the number of FMLP receptors.¹⁵ Although there appeared to be a proportional increase in response of neutrophils to FMLP infusion relative to the extent of injury, this cannot, at least on a statistical basis, explain the decreased numbers of neutrophils 8 hours after injury in rats with 60% burns compared with those with 30% burns. However, there was a significant increase 4 hours after injury in response of neutrophils to ZAS in rats that sustained 60% injury relative to those that sustained 30% injury. Since margination is the initial step²⁹ in directed migration into a wound, the finding that the neutrophils of the rats with greater

injuries were more sensitive to a marginating stimulus but subsequently were present in lower numbers in the wounds was unanticipated. On the other hand, the neutrophils that were more sensitive to ZAS would be more susceptible to "inappropriate" margination in all capillary beds. Although others have not reported changes in wound accumulation of neutrophils, there are reports of margination of neutrophils at sites such as the lung²³ early after 30% burn injury in the rat.

Although previous work⁷ showed that the 30% partial-thickness wounds do not convert to full-thickness injury in the presence of additional 30% full-thickness injury, the possibility remained that the reduced neutrophil migration into the partial-thickness wounds of rats with 60% burns could in part be due to compromised circulation during the acute burn period. In the present study there was no difference in urine output or weight change between rats that sustained 30% partial-thickness burns or this burn plus 30% full-thickness injury. In addition there was no difference in erythrocyte counts between the two groups that were studied serially during the first 8 hours after injury. These findings coupled with lack of evidence of wound conversion to deep injury in the rats with 60% burns and no deaths in either group suggest that significant differences in circulation did not occur between groups.

Thus a depression of the neutrophil response to burn wounds that is directly related to extent of injury has been documented in a model in which susceptibility to infection has already been shown to correlate directly with extent of injury. In vivo evaluation of the circulating neutrophil response to stimuli that induce margination provided a means to study the first phase of neutrophil infiltration into injured tissue in the pathophysiologic environment that exists after major injury. The studies with ZAS, the components of which are held to be major endogenous chemotactic factors, indicated that a relative increase in sensitivity of neutrophils occurs after large burns compared with smaller injuries. That this propensity to margination resulted in subsequent accumulation of less rather than more neutrophils in wounds of rats with large burns suggests and is consistent with in vitro data indicating that a more extensive injury is associated with indiscrete margination at other sites. Such "inappropriate" margination of "sticky" neutrophils has previously been implicated in the development of organ failure after injury.^{23,30,31} However, the increased susceptibility to infection associated with the more extensive injury indicates that the diversion of neutrophils from the wound may have equally serious effects on host defense against infection.

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DISCUSSION

Dr. Solomkin (Cincinnati, Ohio). I think my interpretation of the results is similar to yours except I am not sure whether the zymosan-activated rat serum experiments really show what we would have anticipated: deactivation caused by circulating confluent activation products.

In line with the hypothesis you support of deactivation by circulating confluent products such as C5a, do such deactivation and loss of subsequent responsiveness after presumptively intravascular exposure really suggest that C5a itself is not a tissue mediator or tissue chemotactic but rather that its function may be to serve as some sort of an activating system? I think your results are compatible with the idea that these cells are activated by some substance and that they seem to have lost some sensitivity to C5a while gaining responsiveness.

Dr. Ed Deitch (Shreveport, La.). The neutrophil is the most important cell in the acute response to infection. As far back as the 1950s Miles showed that neutrophils do not get to the wound and are numbered by 4 hours or so. I think there is some clinical relevance in the fact that the larger the wound the fewer the number of cells to get there.

Is there a problem with the absolute number of neutrophils

available to the bone marrow? An alternative question is since the neutrophils' responsibility is to get out of the vessel into the wound, is there any problem with the fact that you are looking at margination inside the vessels rather than at total accumulation of neutrophils?

Professor Mesmer. Do you have some electron microscopic findings to show that there is really intricate contact between the neutrophil and endothelium?

Dr. Flints. Is the problem in the neutrophils or in the vessels? In other words, is the signal coming from the wound to the vessel in the wound to signal the neutrophils to stick or is it something that circulates in the plasma?

Dr. Yurt (closing). With regard to Dr. Solomkin's question as far as responsiveness to C5a and whether it is a circulating factor or wound factor, I agree with him that based on his studies, as well as ours, it looks like C5a may well modulate some of these responses and not necessarily primarily act as a wound mediator.

I would also emphasize that these results seem to correspond to his data showing *in vitro* responses that are somewhat similar to the ones we have shown here. We would also point out in relation to that as well as to Dr. Deitch's question that I think one of the values of this type of assay is that although it may be a little more difficult to decipher exactly what is occurring, it does take into account all the mediators that are involved in this process such as steroids, epinephrine and cell-derived factors. More specifically with regard to Dr. Deitch's question, we have shown that there are equal numbers of neutrophils available in the circulation after each of these injuries but certainly it is possible that part of the findings could relate to total available stores. I do not think it is necessarily one single factor involved although I do feel quite certain that there are functional differences in the neutrophils.

Again, with regard to his comment as far as accumulation in the wound, I want to reemphasize that neutrophils were reported per vessel because we did not want to get into a problem with surface area changes and that at the 8-hour time point a predominant number of these neutrophils were outside the vessel, not marginated at that point, so that the vessel was merely a denominator and should not be taken to indicate that only margination was evaluated.

I think that relates to Dr. Mesmer's question to some extent in that we were looking at infiltration in the later studies. However, we have not done electron microscopy to see if the neutrophils that were marginating after 4 hours were different or were from different populations of neutrophils.

Finally, with regard to Dr. Flint's question, I really cannot answer that with certainty. I think we do have evidence that the neutrophils are responding differently at least to some extent but certainly your point is well taken in that vascular changes have to be taken into account as far as the ability of these cells to first marginate and then gain access into the wound.